

Functional Analysis of Aquaporin-1 Deficient Red Cells

THE COLTON-NULL PHENOTYPE*

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The aquaporin-1 (AQP1) water transport protein contains a polymorphism corresponding to the Colton red blood cell antigens. To define the fraction of membrane water permeability mediated by AQP1, red cells were obtained from human kindreds with the rare Colton-null phenotype. Homozygosity or heterozygosity for deletion of exon I in AQP1 correlated with total or partial deficiency of AQP1 protein. Homozygote red cell morphology appeared normal, but clinical laboratory studies revealed slightly reduced red cell life span *in vivo*; deformability studies revealed a slight reduction in membrane surface area. Diffusional water permeability (P_d) was measured under isotonic conditions by pulsed field gradient NMR. Osmotic water permeability (P_o) was measured by change in light scattering after rapid exposure of red cells to increased extracellular osmolality. AQP1 contributes ~64% ($P_d = 1.5 \times 10^{-3}$ cm/s) of the total diffusional water permeability pathway, and lipid permeation apparently comprises ~23%. In contrast, AQP1 contributes >85% ($P_o = 19 \times 10^{-3}$ cm/s) of the total osmotic water permeability pathway, and lipid permeation apparently comprises only ~10%. The ratio of AQP1-mediated P_o to P_d predicts the length of the aqueous pore to be 36 Å.

It has long been argued whether the fundamental process of membrane water permeability results from diffusion of water through the lipid bilayer, transit of water through protein pores, or the sum of both processes (reviewed by Finkelstein (1987)). Diffusional water permeability (P_d)¹ represents transmembrane flow of water in the absence of an osmotic gradient; human red cells exhibit $P_d \sim 3 \times 10^{-3}$ cm/s at 25 °C (Brahm, 1982). Osmotic water permeability (P_o) represents transmembrane flow of water driven by an osmotic gradient; human red cells exhibit $P_o \sim 20 \times 10^{-3}$ cm/s at 25 °C (Moura *et al.*, 1984). Recognition that red cell water permeability is inhibited by mercurials was taken as evidence that protein water pores must exist (Macey and Farmer, 1970), but it remains uncertain how much membrane water permeability is due to lipid and

how much is due to protein pores.

Discovery of the water transporter AQP1 (CHIP28) in red cells and renal tubules (Denker *et al.*, 1988; Smith and Agre, 1991; Preston and Agre, 1991) led to the identification of the aquaporin family of water transporters (reviewed by Knepper (1994); Chrispeels and Agre, 1994). Analysis of AQP1 cDNA (Preston *et al.*, 1992) and highly purified AQP1 protein (Zeidel *et al.*, 1992b, 1994) permitted molecular characterization of water transport. The structure of aquaporins has been resolved by site-directed mutagenesis (Preston *et al.*, 1993, 1994a; Shi *et al.*, 1994; Jung *et al.*, 1994) and by two-dimensional (Walz *et al.*, 1994a, 1995; Mitra *et al.*, 1994, 1995; Jap and Li, 1995) and three-dimensional electron crystallography (Walz *et al.*, 1994b).

The physiological importance of the collecting duct aquaporin homolog became apparent when mutations in the AQP2 gene were found in some patients with nephrogenic diabetes insipidus (Deen *et al.*, 1994). The Colton blood group antigens (Co^a and Co^b) represent a surface polymorphism in the AQP1 molecule (Smith *et al.*, 1994), and extremely rare individuals (Colton-null) became sensitized to Co^a and Co^b fetal red cells during pregnancy (Lacey *et al.*, 1987). The physiological importance of AQP1 was thrown into question when all three unrelated individuals with the Colton-null phenotype were found to be homozygous for disruptions in the AQP1 gene, yet none suffered an obvious clinical defect (Preston *et al.*, 1994b).

Detailed biochemical and biophysical studies of red cells from homozygous Colton-null individuals and their heterozygous relatives have not previously been performed. Thus, it is not certain if they are entirely normal clinically or how they may compensate for absence of AQP1, the major red cell water transporter. Existence of red cells with a specific deficiency of AQP1 protein should provide the purest system for determining the fractions of diffusional (P_d) and osmotic (P_o) water permeabilities mediated by AQP1 and will permit refined calculation of the length of the aqueous pathway.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal, affinity-purified rabbit antibodies were described (Smith and Agre, 1991). Anti-rabbit IgG was from Boehringer Mannheim; enhanced chemiluminescence reagents were from Amersham Corp.; electrophoresis reagents were from Bio-Rad.

Blood Preparations—Human blood was obtained by institutionally approved venipuncture. Red cells were washed in 3 volumes of phosphate-buffered saline (7.5 mM sodium phosphate, pH 7.4, 150 mM NaCl) at 1000 × *g* for 10 min and resuspended to the original volume just before analyses. Red cell membranes were prepared by hypotonic lysis with chilled 7.5 mM sodium phosphate, pH 7.4, 1 mM Na₂EDTA, 0.2 mM phenylmethylsulfonyl fluoride (Bennett, 1983). Inhibition studies were performed on red cells after a 30-min incubation period at 37 °C in 1 mM PCMBs. SDS-PAGE was performed with 11% acrylamide slabs (Laemmli, 1970). Immunoblots were prepared with enhanced chemilu-

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¹ The abbreviations used are: P_d , coefficient of diffusional water permeability (cm/s); P_o , coefficient of osmotic water permeability (cm/s); PCMBs, *p*-chloromercuribenzenesulfonic acid; PAGE, polyacrylamide gel electrophoresis; AQP1, aquaporin-1.

minescence (Nielsen *et al.*, 1993).

Southern Blot—Leukocyte genomic DNA was isolated by QIAamp (QIAGEN), digested with *Pst*I, electrophoresed into 1% agarose gels, and transferred to nylon membranes (GeneScreen Plus, DuPont NEN). Blots were hybridized with a 32 P-labeled human *AQP1* cDNA (10^6 dpm/ml) corresponding to nucleotides 15–822 (Moon *et al.*, 1993). After 2-h washes at 65 °C in 0.1 SSC, 0.1% SDS, blots were exposed to Kodak XAR-5 film for 4 days.

Cellular Deformability—Osmotic gradient ektacytometry was employed to continuously assess whole cell deformability over a range of osmolalities (Clark *et al.*, 1983). Osmolality at the deformability minimum in the hypotonic region reflects the surface to volume ratio for the whole population of cells. A deformability maximum at 290 mosM reflects membrane surface area. Red cells were exposed to osmolalities for 45 s prior to measuring osmotic deformability profiles over 8 min.

Diffusional Water Permeability (P_d)—Water permeability in the absence of an osmotic gradient was measured by pulsed field gradient NMR (Andrasko, 1976; Kärger *et al.*, 1988). By applying a pair of pulsed field gradients to a stimulated echo sequence, water resonance peak intensity can be sensitized to molecular diffusion. In a system consisting of two compartments (A (internal) and B (external)) with different water diffusivity (D_A and D_B), the signal intensity (S/S_0) obeys Equations 1–4:

$$\frac{S}{S_0} = p_1 e^{-K^2 D_1 \Delta} + p_2 e^{-K^2 D_2 \Delta} \quad (\text{Eq. 1})$$

where

$$D_{1(2)} = \frac{1}{2} \left(D_A + D_B + \frac{1}{K^2} (k_A + k_B) \mp \left[\left(D_B - D_A + \frac{1}{K^2} (k_B - k_A) \right)^2 + \frac{4k_A k_B}{K^4} \right]^{1/2} \right) \quad (\text{Eq. 2})$$

$$p_1 = \frac{1}{D_1 - D_2} (p_A D_A + p_B D_B - D_2) \quad (\text{Eq. 3})$$

$$p_2 = 1 - p_1 \quad (\text{Eq. 4})$$

S and S_0 are water signal intensities in the presence and absence of gradient, k_A and k_B are forward and back rate constants, and p_A and p_B are the mole fraction of spins in each compartment; in $K = \gamma g \delta$, γ is the nuclear gyromagnetic ratio, and g and δ denote gradient strength and length; Δ is the separation between two gradients.

Experimentally, the gradient strength g was changed from 3–55 G/cm, and the signal decay (S) recorded with at least two values of Δ (50 and 100 ms) and simultaneously fitted to Equations 1–4 to obtain four unknowns (D_A , D_B , k_A , and k_B). Fitting was by Powell function (Press *et al.*, 1992). Permeability in cm/s was obtained by multiplying the forward rate constant k_A by volume to surface area ratio (4.57×10^{-5} cm). Diffusion was measured in a General Electric Omega 400 NMR Spectrometer equipped with a triple axis gradient unit (up to 130 G/cm); 5-mm sample tubes were analyzed at 20 °C.

Osmotic Water Permeability (P_f)—Osmotic water permeability was measured at 20 °C (Zeidel *et al.*, 1992b) by abruptly doubling the external osmolality of intact red cells with an equal volume of phosphate-buffered saline-sucrose in a stopped flow apparatus, 0.9 ms dead time (SF.17MV, Applied Photophysics, Leatherhead, UK). Red cell volumes were monitored by light scatter (excitation wavelength 600 ± 1.5 nm, generated with a 150-watt mercury-xenon arc and monochromator, f3.4 grating (Applied Photophysics)); emission wavelength >515 nm was measured through a cut-on filter (Oriel Corp., Stratford, CT). Averaged data from 8 to 16 determinations were fitted to single exponential curves. The P_f was calculated by iteratively solving the water permeability equation using MCAD software (MathSoft, Cambridge, MA),

$$dV(t)/dt = (P_f) \times (SAV) \times (MVW) \times [(C_{in}/V(t)) - C_{out}]$$

where $V(t)$ is relative red cell volume as a function of time, P_f is in cm/s, SAV is the vesicle surface area to volume ratio, MVW is the molar volume of water (18 ml/mol), and C_{in} and C_{out} are initial concentrations of total intracellular and extracellular solute. Red cell radii were calculated from cell volume.

RESULTS

Clinical Laboratory Analysis of *AQP1* Deficiency—Probands from three kindreds with the Colton-null phenotype were re-

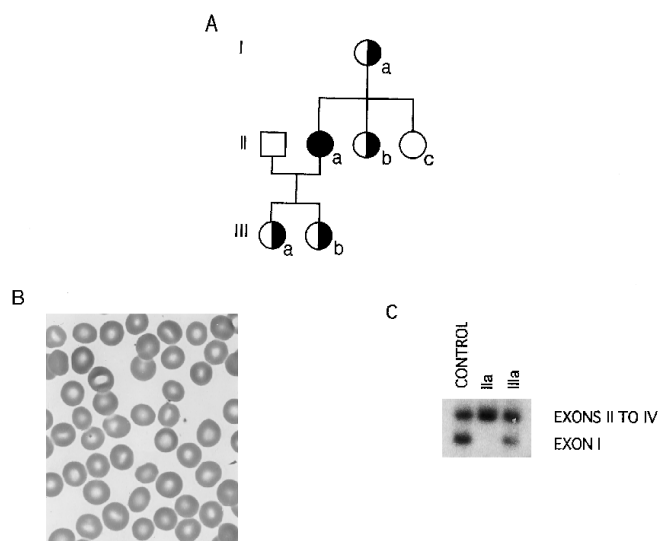


FIG. 1. Genetic and morphologic analysis of *AQP1*-deficient, Colton-null red cell phenotype. A, genealogy of Colton deficiency in three generations of kindred 1; open figures, normal Colton antigens; half-closed figures, reduced Colton antigens; closed figures, absent Colton antigens. B, Wright's stain of peripheral blood from Colton-null subject IIa. C, Southern blot of 10 μ g of genomic DNA obtained from leukocytes of selected members of kindred 1 after digestion with *Pst*I and electrophoresis through 1% agarose, probed with 32 P-labeled *AQP1* cDNA.

cently found to be homozygous for disruptions of their *AQP1* genes: deletion of exon I in kindred 1, frameshift mutation in kindred 2, and missense mutation in kindred 3 (Preston *et al.*, 1994b). Red cells from three generations of kindred 1 (Fig. 1A) and probands from kindreds 2 and 3 were evaluated for abundance of the Co^a antigen by agglutination with specific anti-serum; all three probands lacked Co^a, and presumed heterozygotes from kindred 1 had reduced levels of Co^a (Table I). Hematologic consequences of total *AQP1* deficiency are not severe, since red cell morphology, hematocrit, and hemoglobin levels were all normal (Fig. 1B, Table I). Nevertheless, evidence for subtle hemolysis was noted in two probands who had below-normal haptoglobin levels and minimal elevations in reticulocyte count. Zygosity for members of kindred 1 was confirmed by genomic Southern blot of *Pst*I-digested DNA (Fig. 1C), which showed total absence of exon I in the proband sample, subject IIa (–/–), and partial deficiency of the exon I fragment in the DNA from a presumed heterozygote, subject IIIa (+/–).

Biochemical Analysis of Red Cell Membranes—Hypotonically lysed red cell membranes from kindred 1 were studied by SDS-PAGE and immunoblotting. Red cell membranes from unrelated control (+/+), homozygote (–/–), and heterozygote (+/–) appeared equivalent when stained with Coomassie (Fig. 2). Abundance and mobility of spectrin, anion exchanger/band 3 (AE1), glucose transporter (GLUT1), and red cell urea transporter (UT) were equivalent when assessed by immunoblot. In contrast, *AQP1* was undetectable in membranes of the homozygote (–/–), and membranes from the heterozygote (+/–) were ~50% deficient in the protein, although its mobility was not altered (Fig. 2).

Cellular Deformability—Ektacytometry of red cells exposed to a continuously changing osmotic gradient will reveal abnormalities in membrane deformability, surface area, and the ratio of surface-to-volume (Clark *et al.*, 1983). Since osmotic gradient ektacytometry is dependent upon transmembrane water movement, the technique was utilized to study *AQP1*-deficient red cells. When peripheral red cells from a totally *AQP1*-deficient homozygote (–/–) were compared to red cells from a normal unrelated control (+/+), the deformability maximum at

TABLE I
Clinical laboratory values for AQP1-deficient, Colton-variant red cells

Anti-Co^a titer is the reciprocal of the highest dilution of antiserum at which agglutination of the subject's red cells was still detected.

Value	Controls (+/+)	Kindred 1			Kindred 2 (-/-)	Kindred 3 (-/-)
		Ia (+/-)	IIIa (+/-)	IIa (-/-)		
Anti-Co ^a	32	4	8	0	0	0
Hematocrit	36–47%			42.3	44.2	46.8
Hemoglobin	13–15.6 g/dl			14.5	14.8	15.6
Mean cell vol.	80–100 fl			85.7	91.9	95.6
Reticulocytes	≤1%			0.9	1.2	1.6
Haptoglobin	100–300 mg/dl			180	90	72

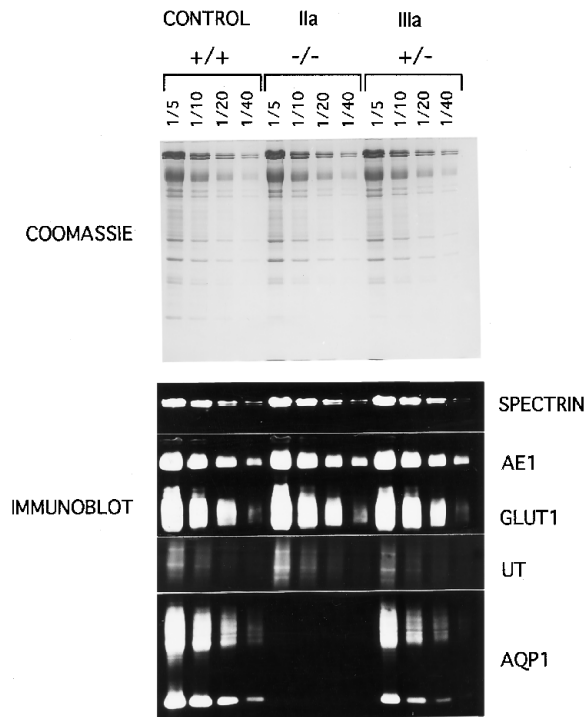


FIG. 2. Biochemical analyses of red cell membrane proteins prepared from an unrelated control individual (+/+), subject IIa who is homozygous for Colton-deficiency (-/-), and subject IIIa who is heterozygous for Colton deficiency (+/-). Top, Coomassie Blue-stained SDS-PAGE slab of red cell membranes. Packed membranes were serially diluted, and 10 μ l were applied to the gel. Bottom, immunoblots of a duplicate SDS-PAGE slab probed with affinity-purified antibodies to spectrin, anion exchanger (AE1), glucose transporter (GLUT1), urea transporter (UT), and aquaporin-1 (AQP1). Note, the AQP1 blot reveals glycosylated subunits (broad bands with slower mobility) and nonglycosylated subunits (sharp bands with faster mobility).

290 mosM/kg was slightly reduced, consistent with a small reduction in membrane surface area (Fig. 3). Also, the deformability minimum in the hypotonic area of the profile was shifted slightly to higher osmolality values, consistent with a modest reduction in surface area to volume. In none of the studies was a major alteration of the profile noted, indicating that the anticipated reductions in water permeability for AQP1-deficient red cells is obscured by the relatively long time frame of this technique. The small reduction in surface area may only reflect the area normally occupied by AQP1 (<3% of total red cell surface area) (Smith and Agre (1991)).

Diffusional Water Permeability (P_d)—The forward rate constant k_A for water diffusion can be computed from the water signal intensity decay curves. Diffusional permeability (P_d) values of red cells from a normal control, two partially AQP1-deficient heterozygotes, and a totally AQP1-deficient homozygote were compared (Fig. 4, A and B). Control red cells had a high value ($P_d = 2.36 \times 10^{-3}$ cm/s) consistent with the value

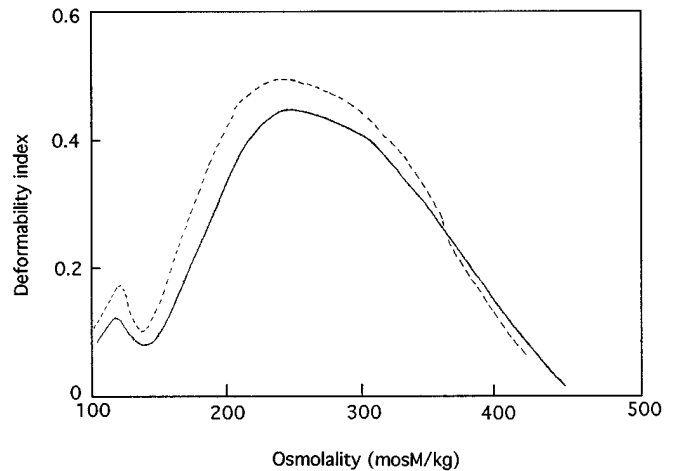


FIG. 3. Osmotic deformability profiles of red cells. Peripheral red cells from an unrelated control individual (+/+, dashed line) and an individual homozygous for AQP1 deficiency (subject IIa, -/-, solid line) were analyzed by osmotic gradient ektacytometry, a technique that determines whole cell deformability while the osmolality of the suspending medium is continuously being changed. Consistent with a small reduction in membrane surface area and a small decrease in surface-to-volume ratio, the Colton-null red cells exhibit a minor reduction in maximum deformability (at ~290 mosM/kg) and a small shift in the osmolality value at which red cells exhibit minimum deformability in hypotonic medium (~140 mosM/kg).

determined by tracer efflux studies (Brahm, 1982). Compared to the control, the homozygote exhibited a P_d value only 36% as large (0.86×10^{-3} cm/s), and the heterozygotes exhibited intermediate values (1.4×10^{-3} cm/s). Although relatively small, the P_d of red cells totally deficient in AQP1 is still significant and appears to reflect the permeability of two pathways (Fig. 4B): (i) one pathway is insensitive to PCMBS ($P_d = 0.55 \times 10^{-3}$; ~23% of total P_d of control) and (ii) the other pathway is inhibited by PCMBS ($0.86 \times 10^{-3} - 0.55 \times 10^{-3} = 0.31 \times 10^{-3}$; ~13% of total P_d of control).

Osmotic Water Permeability (P_f)—When rapidly exposed to a doubling of external osmolality, intact normal red cells shrink to equilibrium in <1 s. Red cells totally deficient in AQP1 exhibited a long time constant, and red cells partially deficient in AQP1 exhibited an intermediate value (Fig. 5A). The osmotic water permeability for control red cells ($P_f = 22.8 \times 10^{-3}$ cm/s) is similar to that previously established (Moura *et al.*, 1984). The P_f measured for the homozygote was significantly lower (3.0×10^{-3} cm/s), while the heterozygote value was intermediate (7.1×10^{-3} cm/s). Addition of PCMBS to all of the red cell samples decreased the P_f values significantly (Fig. 5B).

Temperature Dependence of P_f and P_d —The Arrhenius activation energy measured for normal control red cells ($E_a \sim 5$ kcal/mol, Table II) was similar to the activation energy for diffusion of water in absence of any barrier (4.8 kcal/mol) (Wang, 1965). The activation energy for red cells totally deficient in AQP1 (10–11 kcal/mol, Table II) was close to the value measured for water crossing simple lipid bilayers (12–14 kcal/

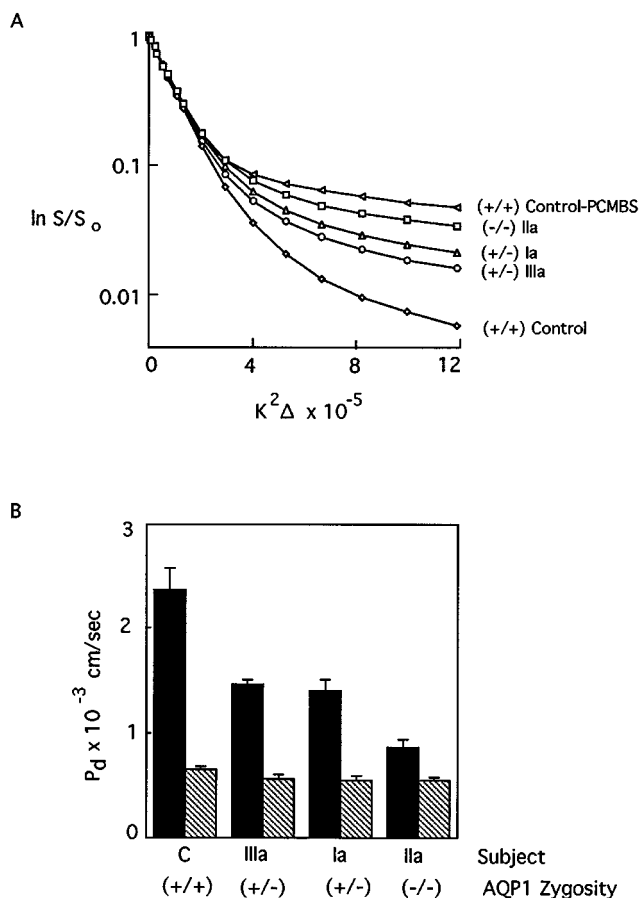


FIG. 4. Diffusional water permeability (P_d) of red cells measured by pulsed field gradient NMR spectroscopy. *A*, Water signal intensity versus $K^2 \Delta$ values for a series of red blood cell suspensions. *B*, coefficient of diffusional permeability values computed from measurements in absence (black bars) and presence of 1 mM PCMBs (stippled bars). Shown are mean values \pm S.D. ($n = 8$ measurements on different control individuals and subject IIa; $n = 4$ measurements of subjects Ia and IIIa).

mol) (Redwood and Haydon, 1969; Price and Thompson, 1969). Activation energies measured for red cells with partial AQP1 deficiency exhibited an intermediate value (6–7 kcal/mol). Water exists mostly as three-dimensional hydrogen-bonded network, and the energy required to break a hydrogen bond is ~ 5 kcal/mol (Stillenger, 1980). The low activation energy for water permeation through AQP1 suggests that the water molecules move through the pore without significant interactions with the walls of the pore.

DISCUSSION

These studies have further defined the biophysical behavior of AQP1-mediated water permeability in red cells. Several years before identification of the aquaporins, indirect studies using mercurial inhibitors suggested that $\sim 90\%$ of the red cell osmotic water permeability and $\sim 50\%$ of diffusional permeability results from transit of water through hypothetical pores in the membrane, while the remainder is due to passage of water through the lipid bilayer (reviewed by Finkelstein (1987)). Red cells totally devoid of AQP1 but lacking other defects provided the membranes needed for direct measurement of non-AQP1-mediated parameters. Our determinations are close to the predicted values, and their significance is underscored by determination of intermediate values from red cells from heterozygotes, although it is not clear why heterozygote values were less than half of the normal values.

Low molecular weight solutes do not permeate through

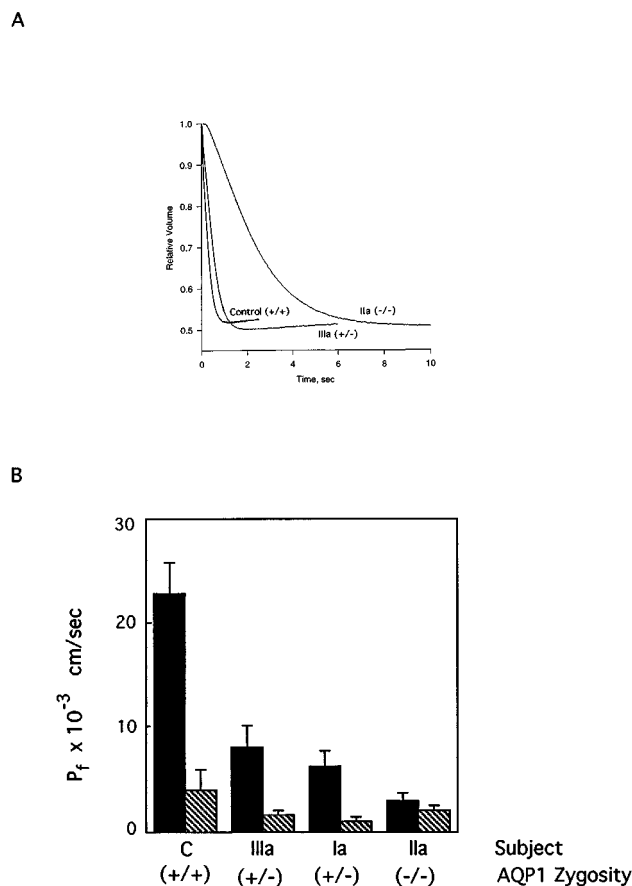


FIG. 5. Osmotic water permeability (P_f) of red blood cells. *A*, representative tracings of red cells abruptly exposed to twice the external osmolality with time course of water efflux monitored in a stopped flow spectrophotometer. *B*, coefficients of osmotic water permeability in the absence (black bars) and presence of 1 mM PCMBs (stippled bars). Shown are mean values \pm S.D. ($n = 6$ measurements of different control individuals; $n = 4$ measurements of subject IIa; $n = 2$ measurements of subjects Ia and IIIa). Note that tracings in panel *A* are exponential fits.

TABLE II
Activation energies of water permeation across red blood cell membranes

Water permeabilities were measured between 10 and 35 °C.

Subject	Osmotic permeability E_a	Diffusional permeability E_a
kcal/mol		
Control (+/+)	4.6	5.6
Heterozygote Ia (+/-)	6.2	6.8
Homozygote IIa (-/-)	10.2	10.8

AQP1 (Zeidel *et al.* 1992a, 1992b, 1994), and urea is now known to be transported across red cell membranes by a protein unrelated to the aquaporins (Olives *et al.*, 1995). Therefore, it is reasonable to conclude that water goes through AQP1 by single file diffusion (reviewed by Finkelstein (1987)). Since the diameter of water is 2.72 Å, the diameter of the aqueous pore should be slightly larger (3–4 Å). It has been theorized (Levitt, 1974) that for a water pore to be sufficiently narrow so that individual water molecules cannot pass each other, the ratio of $P_f/P_d = N$, where N is the number of water molecules in single file within the pore. Water permeability measurements in cells totally deficient in AQP1 permitted correction of P_f and P_d to that specifically mediated by AQP1. The ratio $P_f/P_d = 13.2$ predicts the length of the aqueous pathway of AQP1 to be $13.2 \times 2.72 = 36$ Å, somewhat shorter than the estimated ~ 50 -Å width of the lipid bilayer (Fig. 6). The structure of AQP1 is being investigated by membrane crystallography of function-

SINGLE FILE DIFFUSION OF WATER

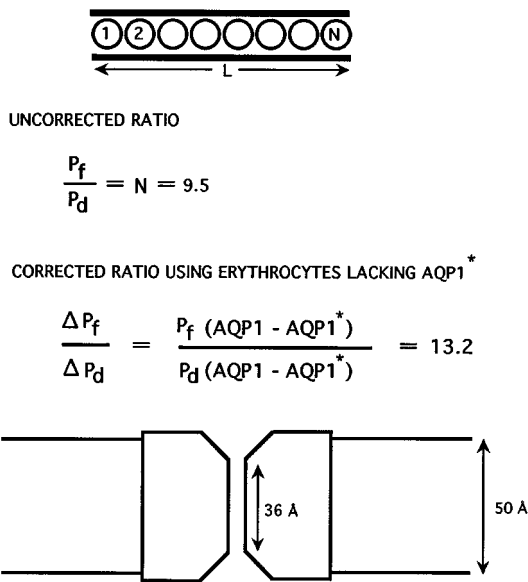


FIG. 6. Schematic of single file diffusion of water through a pore. The equivalent length of the pore (L) is 36 Å (N , the number of water molecules $\times 2.72$ Å, the molecular diameter of water). The assumed width of the bilayer is 50 Å.

ally active molecules (Walz *et al.*, 1994a), and their tetrameric organization has been resolved to <7 Å (Walz *et al.*, 1995; Mitra *et al.*, 1995; Jap and Li, 1995). Studies of site-directed mutant molecules indicate that the tetramer contains four functionally independent pores, although it remains uncertain where the pores reside within the protein (Jung *et al.*, 1994; Preston *et al.*, 1993). Further refinements in the membrane crystallization process may make it possible to visualize aqueous pores, which are predicted to be 4 Å in diameter and 36 Å in length.

These studies also provide further insight into the physiological consequences of red cell membrane water permeability. The surprising observation (Preston *et al.*, 1994b) that total deficiency of AQP1 does not produce obvious clinical manifestations has been confirmed; however, subtle evidence for shortened red cell life span and reduced membrane surface area was observed (Table I, Fig. 3). Red cells from the individuals homozygous for disruptions in the *AQP1* gene do not appear to contain other aquaporins, but measurements of the P_d of these cells revealed a smaller but still significant amount of diffusional water permeability ($\sim 36\%$ of the P_d of control red cells). This non-AQP1-mediated diffusional water permeability may partially explain the lack of hematologic manifestations of the AQP1-deficient state. In contrast, the osmotic permeability measurements of red cells totally deficient in AQP1 was more dramatically reduced ($<15\%$ of the P_f of control red cells). The physiological importance of P_f is thought to involve the need for rapid osmotic water movements when red cells permeate the hypertonic renal medulla, but it remains unknown if AQP1-deficient individuals retain full renal concentration mechanisms.

If osmotic water permeation occurred exclusively through AQP1, $P_f/P_d = 1$ in red cells totally deficient in AQP1. Our studies provided a ratio of 3.4, suggesting that pathways other than AQP1 and diffusion through the lipid bilayer must exist in red cell membranes. This paradigm is supported by analysis of the P_d in red cells totally deficient in AQP1 (Fig. 4), which appears to be comprised of a small PCMBs-inhibitable path-

way in addition to the PCMBs-resistant pathway, and their composite actions exhibit a high activation energy indicating that they are not aqueous pores. While the identities of these two minor water permeation pathways are unknown, it may be speculated that the PCMBs-inhibitable pathway is a protein, and the glucose transporter has been shown to transport water at a very low rate (Fischbarg *et al.*, 1990; Zeidel *et al.*, 1992a). The PCMBs-resistant pathway may represent simple diffusion through the lipid bilayer. Thus, the movement of water across the red cell membrane may reflect the complex behaviors of multiple membrane components in addition to AQP1.

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